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Investigating the Role of rs1899663 G>T Polymorphism of HOTAIR Gene in Susceptibility to Breast Cancer

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ABSTRACT

Background and Objective: Some single nucleotide polymorphisms of the HOTAIR gene are associated with various types of cancer, such as breast, ovarian, colorectal, and lung cancer. The HOTAIR gene product is a long non-coding RNA that is involved in the regulation of gene expression and apoptosis, and is therefore considered an oncogene. This study was conducted with the aim of investigating the rs1899663 G>T polymorphism in breast cancer susceptibility in the northwestern region of Iran.

Methods: In this case-control study, the peripheral blood of 164 breast cancer patients referred to Tabriz hospitals and 172 healthy individuals was collected. After DNA extraction using saturated salt and proteinase K, single nucleotide polymorphism rs1899663 G>T of HOTAIR gene was investigated using tetra-primer ARMS-PCR. The association between this polymorphism and some clinical and pathological features of breast cancer including age, tumor type, tumor grade, tumor size, lymph node involvement and involved side was investigated.

Findings: The frequency of GG, GT, TT genotypes in sick people was 33.4%, 55.6% and 11%, respectively and in healthy people was 51.7%, 42.3% and 6%, respectively. GG (p=0.001) and GT (p=0.029) genotypes showed a significant association with breast cancer. This polymorphism is not associated with any of the clinicopathological features of breast cancer in this population.

Conclusion: Our data show that patients with GT and GG genotypes of rs1899663 G>T polymorphism of HOTAIR gene are more susceptible to breast cancer, while people with TT genotypes have a lower susceptibility to breast cancer development. The results of in silico analyses also show that these single nucleotide polymorphisms can increase the risk of breast cancer.

Keywords: *Cancer, Breast, Single Nucleotide Polymorphism, HOTAIR.*

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Introduction

Cancer has become one of the most important health problems in the world with a high mortality rate (1). Breast cancer is a heterogeneous disease and environmental factors such as low or late pregnancy, family history, early menopause, hormone therapy, alcohol consumption, inactivity and obesity and genetic factors such as mutations in oncogenes, change in gene copy number and epigenetic changes are involved in the creation, development and prognosis of cancer. HOTAIR gene (Gene ID: 100124700) is genetically associated with various cancers such as esophagus, stomach, neuron-glial and breast cancer in different populations (4).

It seems that the expression level of HOTAIR in primary tumors was a predictor of the strength of metastasis and death (5). The HOTAIR gene is located in the 12q13.13 region between the HOXC11 and HOXC12 gene loci and synthesizes a long non-coding RNA (lncRNA) with a length of 2158 nucleotides containing six 2.2 kb exons. Studies show that the HOTAIR gene plays a role in various processes and its increased expression can lead to the activation of epithelial-mesenchymal transition (EMT) pathways and metastasis (6). Moreover, this gene suppresses the expression of tumor suppressor genes such as HOXD10 and PGR and metastasis suppressor genes such as PCDH10 and JAM2, which can indicate the role of this gene in cancer-related processes (7). HOTAIR acts as a guide to suppress the target gene expression, which leads to HOXD gene silencing by regulating epigenetic changes including methylation and demethylation of histones through two protein complexes named LSD1 and PRC2. This process includes the trimethylation of lysine 27 of the H3 protein by the PRC2 complex and also the demethylation of lysine 4 of the histone H3 protein by the LSD1 protein complex (8, 9).

The studies of Vardhini et al. and Wang et al. show that increased expression of the HOTAIR gene suppresses apoptosis and also preserves the survival of breast cancer cells by enhancing cell growth, invasion and migration (10,11). The studies by Rajagopal et al. indicate that the HOTAIR gene is genetically associated with various cancers such as esophagus, stomach, neuron-glial, and breast cancer in different populations (7). Polymorphisms are one of the common genetic variants that have many variations in different racial groups. HOTAIR gene contains several polymorphisms, one of which is rs1899663 G>T (NC_000012.12:53967209:C:A), which is located in intron 2. According to several studies, it seems that the T allele at rs1899663 is more associated with increased risk in stomach, lung and breast cancer than the G allele (12).

The aim of this study is to investigate the association between rs1899663 G>T polymorphism of HOTAIR gene and susceptibility to breast cancer in Northwestern population in Iran. Furthermore, in silico analyses were used to investigate the possible outcomes caused by the rs1899663 G>T polymorphism in changing the binding tendency of transcription factors to HOTAIR gene regulatory regions and structural changes in its RNA.

Methods

In this case-control study, after approval by the Ethics Committee of Tabriz University of Medical Sciences with the code IR.TBZMED.REC.1400.213, peripheral EDTA-anticoagulated blood was collected from 164 patients with breast cancer referred to Noore Nejat Hospital in Tabriz and 172 healthy individuals without family history of cancer in the northwestern region of Iran. During blood sampling, written consent was obtained from these people to use their DNA for the research. Cases that were not among breast tumors or lacked full personal information and pathology were excluded. The samples were matched in terms of

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age and gender. The type and grading of the tumor was diagnosed by a pathologist. The investigated variables in this study were AA, AG and GG genotypes, and A and G alleles.

Genomic DNA extraction: In this study, Salting-out and proteinase K were used to extract DNA from peripheral blood. The quality of the extracted DNA was evaluated by 2% agarose gel electrophoresis in Laboratory of Cell & Molecular Biology of Shahid Madani University, Azerbaijan.

Polymerase chain reaction: TETRA-ARMS-PCR (Tetra Primer-Amplification Refractory Mutation System-Polymerase Chain Reaction) technique was used to examine rs1899663 G>T polymorphism in the study. In this technique, four primers (13) are used simultaneously in one reaction (Table 1). PCR was performed in a total volume of 20 µl, containing 8.5 µl of PCR Master Mix (AMPLIQON, Iran), 8.5 µl of distilled water, 0.7 µl of external primers, 0.3 µl of internal primers and 1 µl of genomic DNA inside a thermocycler (Techne Biometra, England-Germany). The PCR program was carried out for 5 minutes at 94°C for initial denaturation. In order to generate the appropriate number of copies, 40 cycles were considered: 30 s at 94°C, 45 s at 59°C, 30 s at 72°C and a final extension of 4 min at 72°C. Then, in order to evaluate the accuracy of the reaction, the products obtained from polymerase chain reaction were examined on 2% agarose gel.

Table 1. Sequence of primers (13)

Primer type	Primer sequence	Length of reaction product (bp)
General primers (outer forward)	TGAAAGCCACGATCATTTAACATAACCA	457
General primers (outer reverse)	TATCTACGGAGGACTTACCTTATTCCTG	457
Primer T (inner forward)	CCATTATTCCAGTTGAGGAGGGTGAA	226
Primer G (inner reverse)	CCAAAAGCCTCTAATTGTTGTCGCC	284

Statistical analysis: In this case-control study, to evlauate the relationship between alleles and genotypes in control and patient groups, Pearson's chi-square with 95% confidence coefficient (CI) and odds ratio (OR) was used. SPSS version 24 software was used to investigate the relationship between patients' genotypes and their clinicopathological characteristics as well as the mean index, and $p<0.05$ was considered significant.

In silico analysis: In this study, in silico analysis was performed using online applications rSNPBase and RNAsnp (https://rth.dk/resources/rnasnp/), ALIbaba 2.1 (http://gene-regulation.com/pub/programs/alibaba2/) and splice Aid2.

Results

PCR products were loaded on 2% agarose gel and subjects were classified into GG, GT and TT genotypes based on the size and number of fragments that were created. Size marker or DNA marker (cat: YT8501-ARMAN BIOTEC) was loaded in the first and last wells of the gel and 3 colored bands of 100, 500 and 200 bp were more distinct than the other bands (Figure 1).

Correlation between genotype and clinicopathology characteristics of patients: In rs1899663 G>T polymorphism, no significant correlation was found between clinicopathological characteristics of patients (age, tumor size, tumor stage, side of involvement and involvement of lymph nodes) and genotypes (Table 2).

Comparison of the frequency distribution of genotypes between the two groups of patients and control: The frequency of GG, GT, TT genotypes in sick people were 33.4%, 55.6% and 11%, respectively,

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and in healthy people were 51.7%, 42.3% and 6%, respectively. GG (p=0.001) and GT (p=0.029) genotypes showed a significant association with breast cancer (p<0.05) (Table 3).

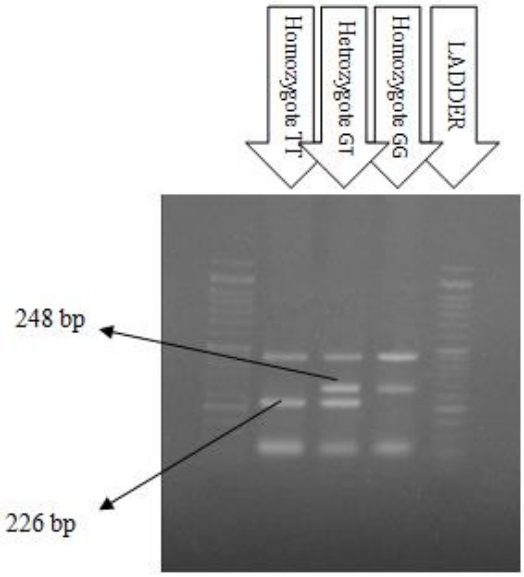


Figure 1. Loading of PCR products on 2% agarose gel. (The LADDER includes 50 bp fragments)

Table 2. Genotypic association between rs1899663 and clinicopathological characteristics of patients

Clinicopathological characteristics	Genotype			p-value
	TT	GT	GG	
Age				
≤45	28	42	7	0.606
>45	12	47	28	
Type of tumor				
Ductal carcinoma in situ	0	4	2	0.647
Invasive ductal carcinoma	17	75	48	
Invasive lobular carcinoma	0	6	1	
Fibroadenoma	0	1	1	
Tumor grade				
I	4	9	10	0.686
II	5	28	20	
III	5	39	17	
Tumor size				
≤2	8	60	36	0.342
>2	5	16	12	
Involvement of lymph nodes				
N0	7	30	27	0.265
N1	5	17	9	
N2	2	18	9	
N3	2	19	5	
Involved side				
Left	8	46	27	0.814
Right	11	42	29	
Both	0	1	0	

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Table 3. Comparison of genotype and allele in patients and controls

Genotype/allele	Patient (n=164) Number(%)	Control (n=172) Number(%)	p-value	OR 95% CI
GG	55(33.4)	89(51.7)	0.001	0.478
GT	90(55.6)	73(42.3)	0.029	1.627
TT	19(11)	10(6)	0.08	2.108
G	200(61)	251(73)	0.098	0.941
T	128(39)	93(27)	0.098	0.941

In silico results: polymorphism analysis of rs1899663 by rSNPBase online software shows that this polymorphism is located near the HOTAIR gene promoter and has a proximal regulatory role and lacks a distal regulatory role that plays a role in regulating the expression of this gene. The RNAsnp online software shows the change in the second structure of RNA, which changes the second structure of RNA to a small amount by changing the polymorphism from G to T state (Figure 2).

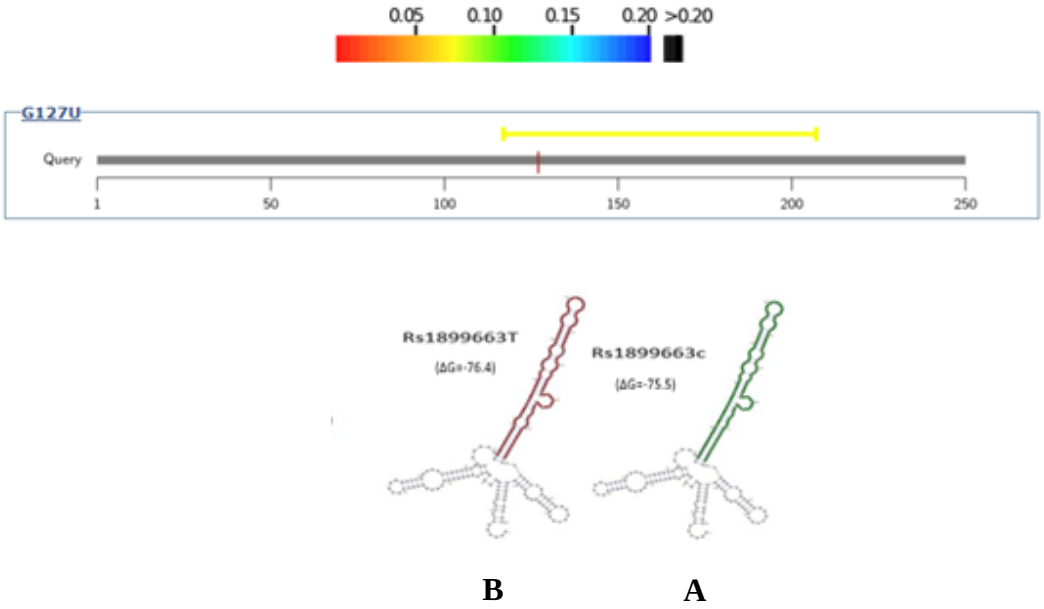


Figure 2. The results of RNAsnp software

The desired polymorphism analysis on HOTAIR gene by ALIbaba online software shows the tendency of different transcription factors to the desired region in wild and mutant state (Table 4).

Table 4. HOTAIR gene polymorphism analysis by ALIbaba software

rs1899663	snp
G	Wild type allele
T	Mutant allele
Sp1, caccc-bi, CREB	Wild-type transcription factors
CREB	Transcription factors in the mutant state

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The results of the desired polymorphism analysis by splisAid2 online software show that this polymorphism binds to RNA in the mutated state to SRPC30 and ETR-3 proteins with a score of -10 to +10. Binding with a positive score facilitates exon splicing and with a negative score facilitates intron splicing (Figure 3).

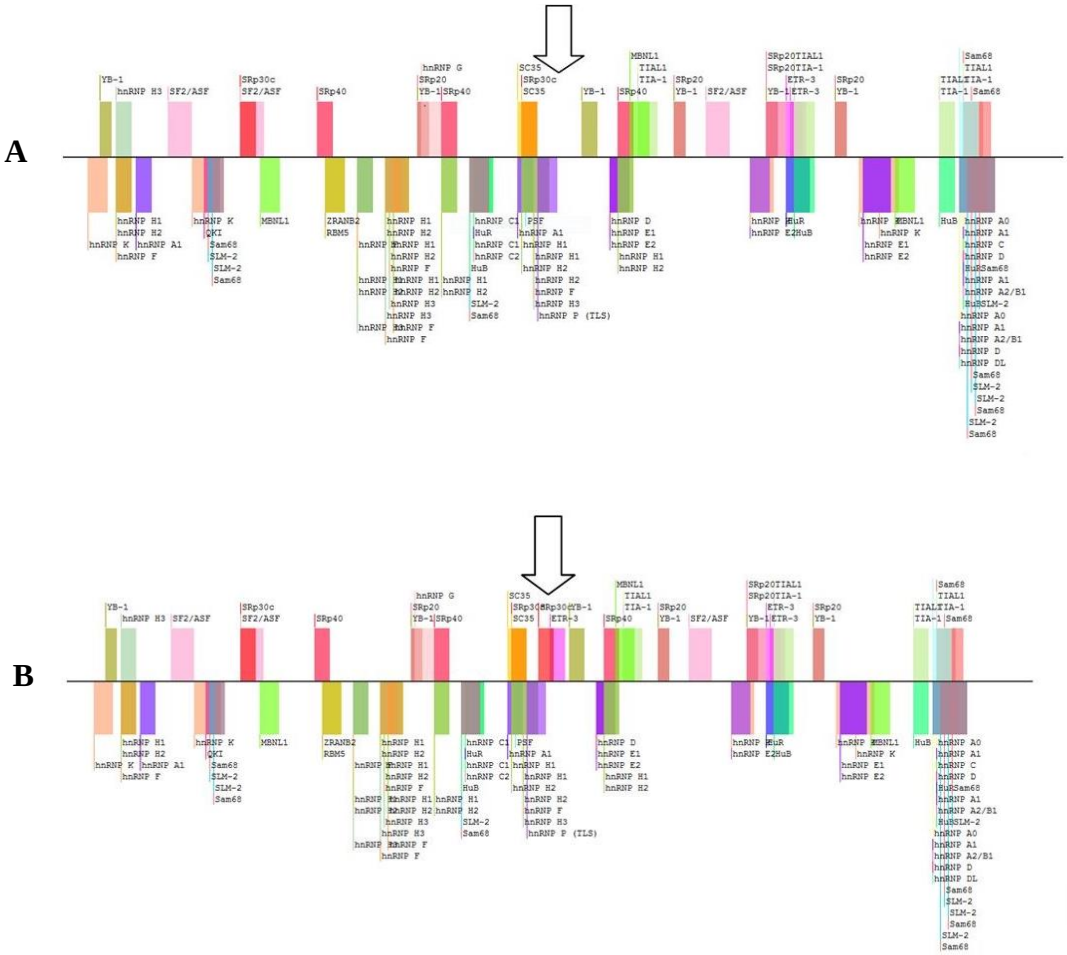


Figure 3. Polymorphism analysis of HOTAIR gene by splisAid2 software

A. non-binding of 2 proteins SRP30C and ETR-3, B. binding of 2 proteins SRP30C and ETR-3 (binding and non-binding of proteins are indicated by arrows)

Discussion

In this study, GG and GT genotypes in single nucleotide rs1899663 G>T polymorphism showed a significant association with breast cancer, but no effective association was found between TT genotype and breast cancer in the population of this region. Although previous studies show that T allele and TT genotype are associated with various cancers (12), our findings do not show an association between T allele and breast cancer. This difference in the results may be due to the different statistical population and may be related to genetic, geographic and racial differences. The results of polymorphism analysis in RNAsnp online software, where the significance level is less than 0.2, show that if the wild allele G is changed to T, a significant change occurs in the second structure of RNA. rs1899663 polymorphism, which was investigated

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in this study, is located in intron 2 of the HOTAIR gene and has an intensifying role (14). In a study conducted by Tian et al., the relationship between HOTAIR gene polymorphisms including rs1899663, rs4759314, and rs920778 and their relationship with cancer development was investigated. In this study, 7151 cancer samples and 8740 control samples were examined, and no significant relationship was observed between HOTAIR gene polymorphisms and the possibility of cancer in them. However, their complementary analyses of HOTAIR rs920778 polymorphism showed that it may be related to the possibility of developing gastrointestinal cancers (15).

The results of a meta-analysis by Lv et al. show that there is a significant relationship between single nucleotide polymorphisms of the HOTAIR gene and the possibility of cancer. However, the prognostic significance of this gene in susceptibility to various types of cancer was not discussed (16). In another study, different HOTAIR gene polymorphisms were investigated. In this study, the association between rs1899663 G>T polymorphism and the risk of breast cancer was observed, but no significant association with other types of cancers was observed in other polymorphisms (17). Various studies in Iran show that HOTAIR gene polymorphisms are associated with thyroid (13), colorectal (18) and other diseases (19, 20), which shows the need for further studies.

According to the results of this study, the GG and GT genotypes in single nucleotide rs1899663 G>T polymorphism are associated with the risk of breast cancer and therefore can be studied as a marker for the prognosis of breast cancer in the population of this region. No significant association was found between this polymorphism and patients' age, tumor size and tumor side, tumor grade and lymph node involvement in the studied population. It is suggested to investigate more polymorphisms of the HOTAIR gene and genes that have a close interaction with it or act in a mutual signaling pathway. Furthermore, this polymorphism should be investigated in other regions of Iran according to geographical latitude and race, both of which influence the outcome of such studies.

Conflict of interest: In this study, there is no conflict of interest with individuals and organizations.

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